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MINIREVIEW

Trimethoprim and Sulfonamide Resistance

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INTRODUCTION

Trimethoprim (TMP) and sulfonamides (SULs) are synthetic antibacterial agents. The first SUL compounds were used in 1932, whereas TMP is a relatively new compound first used in 1962 in England (69). Since 1968, TMP has been used in combination with SULs because the combination of TMP-SULs was supposed to be synergistic in vitro (18). However, clinical experience suggests that TMP-SUL combinations have no clear synergism in vivo (2, 16, 112). In the 1970s, TMP alone came into use, first for the prophylaxis of urinary tract infections and later for the treatment of acute urinary tract infections as well (81).

TMP and SULs share both a wide antibacterial spectrum including common urinary tract pathogens (*Escherichia coli* and other members of the family *Enterobacteriaceae*), respiratory tract pathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae*, and in combination, *Moraxella catarrhalis*), skin pathogens (*Staphylococcus aureus*), as well as certain enteric pathogens (*E. coli* and *Shigella* spp.).

Because of the wide range of clinical indications, TMP-SUL combinations have been used extensively everywhere in the world. In addition, both compounds are relatively inexpensive, a fact that allows for the use of these drugs outside of developed countries.

Today, the most important fear is the development of bacterial resistance to TMP and SULs. To counter bacterial resistance it is essential to understand the molecular background of resistance mechanisms. Analysis of TMP and SUL resistance determinants in clinical bacteria has already revealed new recombination mechanisms that have an impact on spread of the resistance in general. As synthetic antimicrobial agents, TMP and SULs are also examples of agents that bacteria have not met previously, and to which they can develop resistance; this excludes resistance mechanisms related to antibiotic-producing organisms.

In this minireview, we describe the current knowledge of TMP and SUL resistance in major bacterial pathogens and review the TMP and SUL resistance mechanisms.

CONSUMPTION OF TMP AND SULs IN HUMAN AND VETERINARY MEDICINE

Antibacterial agents are powerful selectors for resistance genes in bacterial populations, and the selection pressure is related to the total distribution of antibacterial drugs. General figures for the distribution of TMP and SULs throughout the world are difficult, if not impossible, to obtain. Sales figures are available for countries like Finland and Sweden, which have centralized drug distribution systems.

SULs as single drugs were heavily used in both countries during the first and middle years of the 1970s, but more so in Finland than in Sweden (4.4 compared with 2.1 defined daily doses [DDDs] per 1,000 inhabitants per day in 1976; the DDD for TMP is 0.4 g and that for SUL is 4 g; Fig. 1). The SUL distribution then decreased rapidly and reached zero in Sweden in 1986 and was down to 0.1 DDD per 1,000 inhabitants per day in Finland by 1992. The sales of TMP-SULs reached a peak in Sweden in 1983 and 1984 at 1.3 DDDs per 1,000 inhabitants per day and in Finland in 1985 at 2.4 DDDs per 1,000 inhabitants per day. The use of TMP alone reached a steady level of 0.5 to 1.5 DDDs per 1,000 inhabitants per day in both countries at the beginning of the 1990s.

Figures for the veterinary use of SULs in Sweden show the sales of 6,600 kg for this purpose in 1980 and 2,198 kg in 1989. If these figures are recalculated to human DDDs per 1,000 inhabitants per day they amount to 3.8 for 1980 and 1.2 for 1989, corresponding to a much larger total SUL distribution for veterinary than for human purposes. The corresponding figures for TMP were 134 kg in 1980 and 285 kg in 1989, corresponding to 0.1 and 0.2 DDD per 1,000 inhabitants per day in 1980 and 1989, respectively.

SPREAD OF TMP AND SUL RESISTANCE

When bacterial resistance figures in different studies are compared, attention should be paid to the sources of the isolates and the susceptibility testing methods used (69). Although data for hospitalized patient and outpatient populations should be reported separately, this division has not always been done. Wide variations in bacterial resistance can be observed in different geographical areas and, even in the same area, can depend on the material studied. In addition, recording of data for repeat samples from the same patient may influence records of resistance levels (68).

Urinary tract pathogens. In the 1970s, TMP resistance in isolates of *E. coli* from urine rarely occurred in more than 10% of the isolates from outpatients (31, 51, 53, 71, 133). However, reports from the 1980s showed an increasing incidence of TMP resistance in *E. coli*. The resistance often reached levels of 15 to 20% (17, 46, 51, 54, 58, 63, 98). Resistant gram-negative bacilli are easily transferred by person-to-person contact, as has been shown in day-care centers (132) and nursing homes (184).

Resistance rates of gram-negative pathogens in developing countries have been reported to be clearly higher than those in the developed world; TMP resistance has been reported at high levels of 25 to 68% in South America, Asia, and Africa

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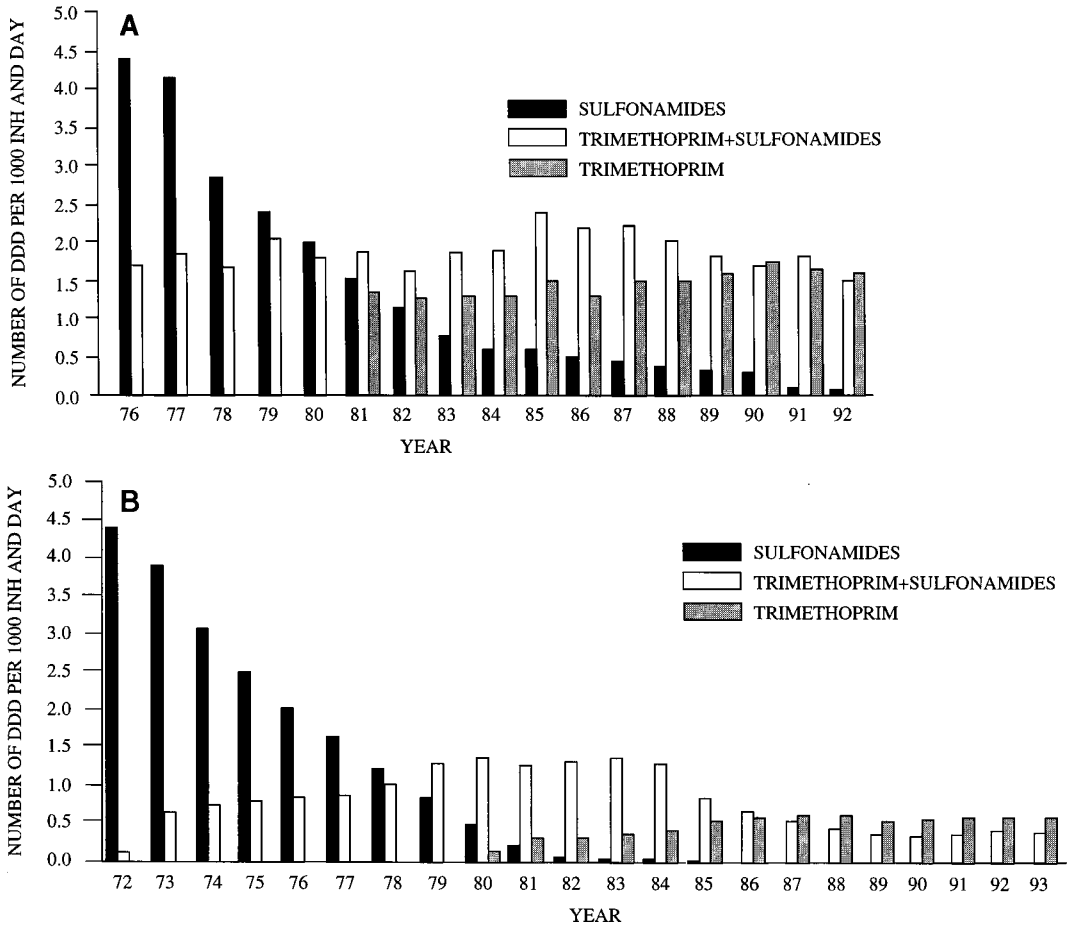

FIG. 1. Drug sales from the Finnish Committee on Drug Information and Statistics, Helsinki, Finland (A), and the SWEDIS computer base of the Medical Products Agency, Uppsala, Sweden (B). Drug consumption figures are based on sales and are expressed as DDDs per 1,000 inhabitants per day.

(89, 108, 177, 187). Also, in parallel, the level of fecal carriage of TMP-resistant enterobacteria has been shown to be high in developing countries (90). These resistant isolates are also easily transferred by travelers not exposed to antimicrobial agents (109, 110).

The difference in rates of resistance between isolates from developed countries and those from developing countries is also shown by studies of the international WHONET surveillance program. Resistance to TMP-SULs among more than 20,000 *E. coli* and 5,000 *Klebsiella pneumoniae* isolates has been registered over the last 2 to 4 years (113). These data show that resistant *E. coli* and *K. pneumoniae* in South and Central America as well as in Asia are far more common than in the United States and Sweden. Only 38 to 59% of the *E. coli* isolates and 47 to 77% of the *K. pneumoniae* isolates from Latin America and Asia are susceptible, whereas the corresponding figures for the United States and Sweden are 87 to 93% and 77 to 91%, respectively.

Although there are not sufficient amounts of clinical data on the usage of TMP-SULs in the treatment of urinary tract infections caused by *Enterococcus faecalis*, failure of TMP-SUL treatment against experimental enterococcal endocarditis speaks against the use of TMP-SULs, at least against severe enterococcal infections (48). SUL alone is ineffective against enterococci (70). Until more evidence is accumulated, TMP-SULs or TMP alone should not be used as a first-line drug in the treatment of enterococcal urinary tract infections (107). Until now, TMP resistance in enterococci has not been transferable (43). As opposed to β-lactamase genes, enterococci and staphylococci do not seem to share the same TMP resistance genes (43).

In *Staphylococcus saprophyticus*, TMP resistance has been reported in only 2% of 96 isolates studied (137). In our own material, 1% of 186 isolates studied were resistant to TMP-SULs and 9% were resistant to TMP (72). More studies are needed to establish the rates of TMP and SUL resistance in *S. saprophyticus.*

Enteric pathogens. TMP-SUL has been the drug of choice in the treatment of shigellosis. However, this is not the case anymore (136). An increase in TMP resistance among *Shigella* spp. is one of the most illustrative examples of the spread of TMP resistance (Fig. 2). This development has also had a clinical impact since SUL resistance in *Shigella* spp. has continuously been at a high level of from 42 to 100% (7, 10, 11, 49, 59, 64, 168, 180).

In the 1970s and the early 1980s, TMP resistance occurred in only a minority of *Shigella* isolates (8, 11, 21, 50, 55, 64, 95, 116, 122). In 1983 and 1984, about 4 to 17% of the isolates were TMP or TMP-SUL resistant (21, 50, 64), in 1985 7 to 21% were resistant, and later, TMP resistance even increased to 52%, depending on the *Shigella* sp. (11, 60, 64, 93, 168, 180). Most of the isolates were multiresistant, with resistance to ampicillin, tetracycline, chloramphenicol, and streptomycin also occurring (49, 59). In addition to *Shigella* spp., enterotoxigenic *E. coli* has been shown to have increased rates of TMP resistance (7, 21).

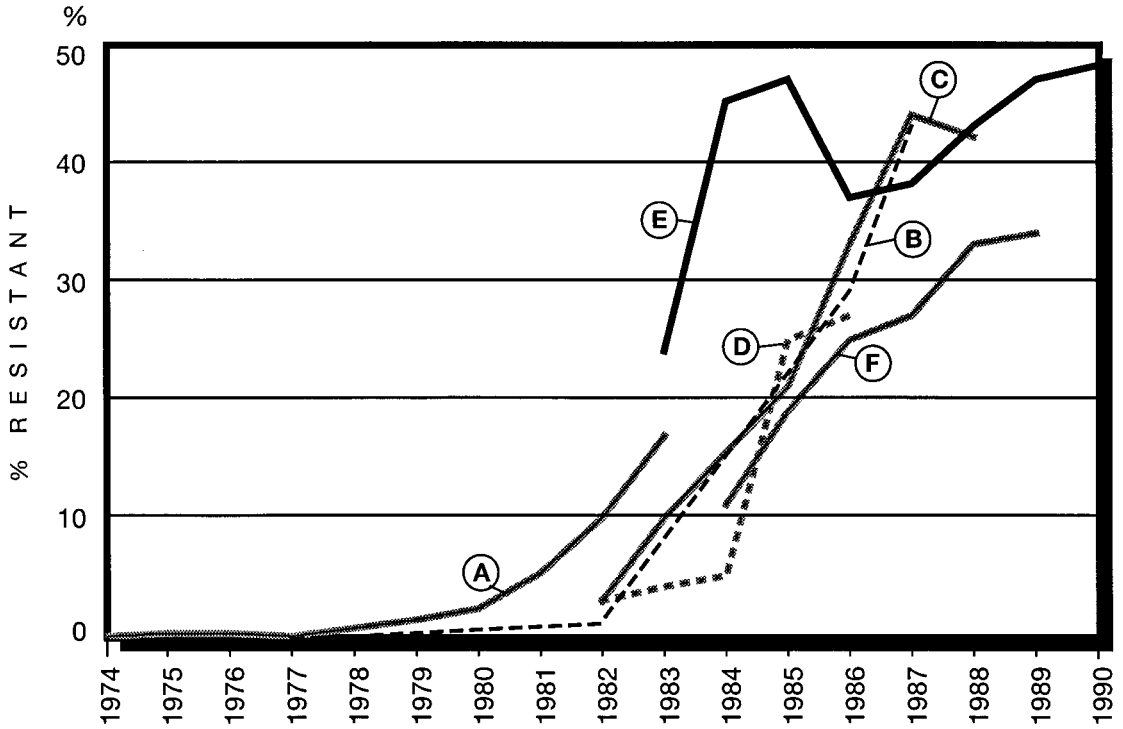

FIG. 2. Development of TMP or TMP-SUL resistance among *Shigella* spp. in different parts of the world. The following letters denote the indicated references: A (50), B (11), C (64), D (21), E (10), F (180).

In *Salmonella* spp., TMP or SUL resistance has not spread as successfully as it has in *Shigella* spp. SUL resistance is fairly common (10 to 75%) among *Salmonella typhimurium* strains collected from cattle, poultry, sheep, and pigs (186). In cattle in England and Wales, TMP resistance occurred in 15% of the *S. typhimurium* isolates examined in the early 1980s, but this increased to 40 to 65% in 1985 to 1987. Isolates from poultry were much more susceptible; only fewer than 5% of the isolates were TMP resistant, and fewer than 20% were resistant to SULs. In other *Salmonella* spp. the rate of resistance has been reported to be much lower (1 to 11%) (186). Although high levels of resistance were reported among isolates from animals in England, the same phenomenon was not seen in isolates from humans in the same geographical area (183). In 1981 and 1988, the levels of SUL and TMP resistance in *S. typhimurium* were 26 to 30% and 8 to 11%, respectively. In *Salmonella enteritidis* and *Salmonella virchow* the corresponding resistance figures were much lower, 2 to 14% and <1 to 9%.

In Spain, TMP-SUL resistance in non-*Salmonella typhi* spp. has been low (<1 to 8%) (105). Low levels of SUL and TMP resistance have also been reported in New Zealand (61). However, outbreaks of TMP-resistant *S. typhi* have been described in India (153, 176).

Other enteric bacterial pathogens like *Yersinia* spp. and *Aeromonas hydrophila* have been reported as being susceptible to TMP-SULs (87, 88, 124). *Campylobacter* spp. or *Helicobacter pylori*, conversely, are not susceptible (47, 181).

Respiratory tract pathogens. TMP-SULs have widely been used in the treatment of respiratory tract infections. Thus, the susceptibilities of the major respiratory tract pathogens *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* are of interest. In England and Scotland, 1.4 and 1.5% of the *H. influenzae* isolates, respectively, were resistant to TMP or SUL in 1981; in 1991, the corresponding figures were 6.8 and 17% (125). In a collaborative European study, TMP-SUL-resistant *H. influenzae* isolates were most common in Spain (41%) and Italy (12%), whereas in all other countries resistance occurred at a rate of less than 8% (82). In the United States, Canada, and Australia, TMP-SUL resistance rates have been low (less than 5%) (23, 45, 78). In *H. influenzae* type b, the rate of resistance to TMP or SUL has been lower than that in nontypeable strains (23).

Although multiresistant *S. pneumoniae* isolates are also usually resistant to TMP-SUL (106), penicillin-susceptible *S. pneumoniae* has retained its susceptibility (79, 148); fewer than 5% of the isolates are resistant. For *M. catarrhalis* (36, 79, 182) resistance rates have been even lower than those for *S. pneumoniae* and *H. influenzae.*

Staphylococci. TMP and SUL resistance also occurs widely among staphylococci. Resistance is spread especially in nosocomial isolates, like methicillin-resistant *S. aureus* and coagulase-negative staphylococci (85, 172). In an international collection of strains, 28% of methicillin-resistant *S. aureus* isolates were TMP resistant and 35% were SUL resistant (172). Of the methicillin-resistant *Staphylococcus epidermidis* strains the corresponding resistance figures were 69% for both TMP and SUL.

In outpatients, although 42% of 64 untreated subjects in England carried TMP-resistant coagulase-negative staphylococci, resistant isolates were a minority of the staphylococcal population (26). However, this low number of resistant isolates may be large enough to heavily colonize the skin during a TMP treatment (73).

MECHANISMS OF RESISTANCE

Chromosomal TMP resistance. Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate-NADP oxidoreductase; EC 1.5.1.3) is an essential enzyme in all living cells. By its structural

analogy to dihydrofolate, TMP is a competitive inhibitor of this enzyme in bacteria. The human DHFR is endogenously resistant to TMP, which is the basis for its selectivity and its clinical use.

The natural occurrence of chromosomal resistance to TMP could conceivably be of three types. One of these would, strictly speaking, include the chromosomal location of transposon Tn*7* (62, 92). Second, low-level resistance to TMP was found to develop from a mutational loss in bacteria of their ability to methylate deoxyuridylic acid to thymidylic acid, making them dependent on an external supply of thymine (52, 83, 99). This mutation relieves the cellular DHFR from its major task of regenerating the tetrahydrofolate involved in the formation of N^5,N^{10}-methylenetetrahydrofolate, which is oxidized in the deoxyuridylate methylation process. In this situation the cell can thus afford to have a relatively large fraction of its DHFR inactivated by TMP. Third, chromosomal resistance to TMP could be due to mutational changes in the gene for DHFR, increasing the K_i for the drug. Such changes could be combined with regulatory mutations, leading to the cellular overproduction of the enzyme and very high levels of TMP resistance.

The most recent example of this third type of chromosomal resistance was found with clinical isolates of *H. influenzae* (32, 34, 126). The MICs of TMP varied between 10 and 200 mg/liter for resistant isolates in comparison with MICs of <0.5 mg/liter for susceptible strains. In other species such intermediate levels of resistance usually reflect chromosomal mechanisms of resistance, and this was also shown to be the case with the *Haemophilus* isolates, which showed overproduction of altered chromosomal DHFR (32–34). Later cloning and sequencing work comparing susceptible and resistant *Haemophilus* strains showed changes in the −35 promoter sequence to make it homologous to that of *E. coli* mutants overproducing DHFR. Mutational changes in the structural gene were also observed as a change of a glutamic acid to a lysine at position 69 and the change of an aspartic acid to an asparagine at position 77. The total length of the structural gene amounted to 160 codons corresponding to a polypeptide of 17,760 kDa (35). A combination of a structurally changed enzyme and overproduction seems to be required for high MICs (39). Biochemical studies on highly purified DHFRs from resistant strains of *H. influenzae* showed K_m values for dihydrofolate and NADPH that were similar to those for similarly purified enzyme from susceptible strains. The concentration of TMP necessary for 50% inhibition of DHFR activity (IC_{50}), however, was found to be 1 nM for an enzyme from a susceptible strain in comparison with 100 and 300 nM for two resistant strains, respectively. Thus, the mutational changes in the DHFRs from resistant strains seemed to specifically decrease the affinity for the inhibitor, TMP, while leaving activity with the substrates NADPH and dihydrofolate intact.

A clinical isolate of *E. coli* was found to be highly resistant to TMP (MIC, >1 g/liter) by combining a several hundredfold overproduction of the chromosomal DHFR with an increase in the K_i for the drug (39). Enzyme overproduction was explained by a promoter up-mutation in the −35 region, a 1-bp increase in the distance between the −10 region and the start codon, several mutations leading to an optimal ribosome-binding site, and several mutations in the structural gene giving more frequently used codons. A mutational substitution of a glycine for a tryptophan at position 30 was thought to relate to the observed threefold increase in the K_i for TMP. All of these mutational changes comprise a remarkable evolutionary adaptation to the presence of antifolates.

Chromosomal mutations to TMP resistance in *E. coli* were first studied and mapped in the laboratory almost 20 years ago (12). In parallel, spontaneous chromosomal antifolate resistance was studied in *S. pneumoniae* (141). Both spontaneous and mutagenically induced chromosomal mutants to TMP resistance were studied in the laboratory by Sheldon and Brenner (138) and Smith and Calvo (146). In all cases studied TMP resistance could be explained either by overproduction of the target enzyme, DHFR, or by alteration of the enzyme, or a combination of both mechanisms. The nucleotide sequence of the *E. coli* gene coding for DHFR was given by Smith and Calvo (145). It consists of 477 nucleotides corresponding to a polypeptide of 159 amino acids and with a relative G+C content of 53%.

Endogenous resistance to TMP occurs among many different bacterial species. DHFRs from lactobacilli are thus less susceptible to TMP than the enzyme of *E. coli*, and the reductase from *Pediococcus cerevisiae* requires a 1,000-fold higher TMP concentration for 50% inhibition (173). Anaerobic bacteria like *Bacteroides fragilis* and *Clostridium* spp. are rather insusceptible to TMP. Purified preparations of DHFR from these strains required several hundredfold to 1,000-fold higher TMP concentrations than a similar preparation from *E. coli* for a 50% inhibition (170).

Chromosomal SUL resistance. The enzyme dihydropteroate synthase (DHPS; EC 2.5.1.15) catalyzes the formation of dihydropteroic acid in bacterial and some eucaryotic cells, like *Pneumocystis carinii* (179), *Toxoplasma gondii* (123), and *Plasmodium falciparum* (192). This enzyme activity is not present in human cells. SUL drugs act as competitive inhibitors of DHPS, thereby blocking folate biosynthesis in the bacterial cell (14). SULs are structural analogs of the normal substrate *p*-aminobenzoic acid and can indeed work as alternative substrates to produce a sulfa-containing pteroate analog (134, 162).

Chromosomal mutations in the *dhps* gene can be isolated in the laboratory (118, 142). An example of this is the nucleotide sequence of the *dhps* gene from *E. coli* C, in which two identical mutants differed from the wild-type strain by a single base pair (163, 164). This change alters the phenylalanine residue at position 28 in the wild type to an isoleucine residue in the mutant. Another mutation altering the same amino acid has also been reported (30).

In *Neisseria meningitidis*, clinical SUL resistance has been found to be linked to the bacterial chromosome (86, 128). Sequence determinations of the resistance determinants from several strains revealed two classes of variants. Most of the clinically isolated SUL-resistant strains of *N. meningitidis* were found to contain a *dhps* gene that was about 10% different from the corresponding gene in SUL-susceptible isolates. It was therefore concluded that the resistance had been introduced by recombination rather than by point mutations. In the first isolate studied, MO035, the whole *dhps* gene showed differences in comparison with those from susceptible isolates. Another isolate, isolate 418, showed identity to the susceptible isolates in the N-terminal and C-terminal parts of the protein (128). Only the central part had a sequence identical to that of the resistant isolate MO035. One distinguishing feature of these resistance genes is an insertion of six nucleotides generating an insertion of two amino acids in a part of the enzyme that is highly conserved. Removal of these two amino acids by site-directed mutagenesis restores the susceptibility (163).

Lopez et al. (94) have studied the *dhps* gene (*sulA*) of *S. pneumoniae* and also located a duplication of two amino acids in a SUL-resistant strain. This duplication is in another part of the protein compared with that in *N. meningitidis*. Finally, the *dhps* gene of a SUL-resistant strain of *Bacillus subtilis* was

TABLE 1. Transferable TMP and SUL resistance traits in gram-negative bacteria

Family	Type, gene	Cassette (length [bp])	Amino acid at position 27	Polypeptide length (bp)	Plasmid or transposon	Reference	Hybridization probe used (reference)
TMP resistance							
1	I, *dhfrI*	Yes (577)	Glu	157 × 2	Tn*7*	42, 111, 139	5′-TTTAGGCCAGTTTTTACCCAAGACTTCGC-3′ (160)
					pLMO150	159	0.49-kb *Hpa*I (159)
1	V, *dhfrV*	Yes (568)	Glu	157	pLMO20	156	5′-GAGAAACATTGCCCATGGCCTCTACGCTC-3′ (160)
							5′-CCTGGACGGCCGATAATGACAACGTAATAG-3′ (191)
							0.49-kb *Hpa*I (156)
1	VI, *dhfrVI*	Yes (606)	Glu	157	pUK672	188	
1	VII, *dhfrVII*	Yes (617)	Glu	157	Tn*5086*	154, 160	5′-AGTGTCGAGGAAAGGAATTTCAAGCTCA-3′ (160)
							0.31-kb *Eco*RV (160)
1	Ib, *dhfrIb*	Yes (>523)	Glu	157	Tn*4132*	189, 191	5′-GTTGGACATCAAATGATGACAATGTAGTTG-3′ (191)
							0.49-kb *Hpa*I (191)
2	IIa, *dhfrIIa*	Yes (484)		78 × 4	R67	13, 152, 154	5′-GATCGCGTGCGCAAGAAAT-3′ (64)
2	IIb, *dhfrIIb*	Yes (384)		78 × 4	R388	156, 167, 193	
2	IIc, *dhfrIIc*	Yes (408)		78 × 4	Tn*5090*	40, 129	0.28-kb *Nhe*I-*Eco*RI (64)
	III, *dhfrIII*	No	Asp	162 × 1	pAZ1	41, 80	0.85-kb *Eco*RI-*Hin*dIII of pFE1242 (64)
	IV	ND[a]	Asp	ND	pUK1123	174, 190	
	VIII, *dhfrVIII*	No	Asp	169	Tn*5091*	154, 154b	5′-CTAACGGCGCTATCTTCGTGAACAACG-3′ (154)
	IX, *dhfrIX*	No	Asp	177	pCJO01	77	5′-AGTGTCGAGGAAAGGAATTTCAAGCTC-3′ (75)
	X, *dhfrX*	No	Asp	182	pDGO100	117	
	XII, *dhfrXII*	Yes (584)	Glu	165	pEH1	65, 140	
	IIIb	ND	Asp	ND	pBH600	9, 173b	
	IIIc	ND	ND	ND		9	
SUL resistance							
	I, *sulI*	No		279	Tn*21* R388	156	0.66-kb *Sac*II-*Bgl*II (131)
	II, *sulII*	No		271	RSF1010 pGS05	130	0.78-kb *Hin*cII (131)

[a] ND, not determined.

identified as a part of an operon containing several other genes involved in folic acid biosynthesis (144).

Plasmid-borne TMP resistance. Plasmid-mediated TMP resistance was first described in 1972 (38) and is caused by nonallelic and drug-insusceptible variants of chromosomal DHFR (4, 143). The genes for some of these enzymes may be temporarily located on the chromosome by virtue of transposon movement, but they are still referred to as plasmid-borne or transferable DHFRs.

(i) Numerous types of plasmid-borne DHFR. The two initially observed plasmid-borne DHFRs mediating resistance to TMP were found by Pattishall et al. (119) to be distinct from each other. At present 16 different types have been found in gram-negative facultative rods (169), and most of these have been defined by amino acid sequence analysis, nucleotide sequencing, or both (Table 1). The gene for one additional type of transferable DHFR, S1, in Tn*4003* of *S. aureus* has been sequenced and the enzyme has been purified (29, 135). The accessory DHFRs show different enzyme characteristics and confer different MICs on their hosts. The most rational way to classify TMP resistance genes is according to amino acid sequence.

Phylogeny analysis has revealed that two subgroups of the transferable DHFRs are related (64 to 88% identity) significantly over the background level (20 to 40% identity in different species) and here are called families (74, 154b). The first of these, family 1, includes enzyme types I, V, VI, VII, and Ib (42, 139, 156, 160, 188, 191). These DHFRs are 64 to 88% identical,

and the polypeptide length is invariably 157 amino acids. At least the type I enzyme is homodimeric (111). All proteins of family 1 mediate resistance to very high levels of TMP (⩾1 g/liter), whereas the IC_{50}s vary substantially and range from 10^{-6} to 10^{-4} M TMP. Also, the family 1 DHFRs carry a glutamate corresponding to the aspartate residue at position 27 of the chromosomal *E. coli* enzyme (Table 1) (5, 160). A glutamate at that position is found in all vertebrate DHFRs, but among procaryotic and yeast enzymes it is found, beside those of family 1, only in DHFR of type XII (65, 140).

The family 2 DHFRs include types IIa, IIb, and IIc, which are completely unrelated to other DHFRs in procaryotes and eucaryotes but which are closely related to one another (78 to 86% amino acid identity) (40). The polypeptides of family 2 are much shorter (78 amino acids) than those of all other DHFRs (150 to 200 amino acids), and the active enzyme is homotetrameric. Most enzyme characteristics differ from those of other DHFRs, and these enzymes are extremely insusceptible to TMP (IC_{50}s, >1 mM) (147). The amino acid sequence and the three-dimensional structure of type IIa DHFR have been determined (103, 152).

The plasmid-borne DHFRs in gram-negative bacteria other than those of family 1 and family 2 are far less related to each other, having only 20 to 50% amino acid sequence identity. The similarity between plasmid-borne and chromosomal DHFRs in different bacteria including *E. coli* (145) is in a similar range (154b). There are three-dimensional structure data for *E. coli* DHFR either free or bound to inhibitors and substrates (101, 102). The closest relation to *E. coli* DHFR is shown by the type III enzyme (about 50%), which is monomeric (80). This moderate degree of similarity could be compared with that of enzyme S1 of Tn*4003*, which is 80% identical to the chromosomal enzyme of its host, *S. aureus* (29, 135). The type III and S1 enzymes are the only accessory DHFRs for which there is a clue to their origins. DHFR of type III, as well as enzymes of types IIIb, IIIc, IV, and type IX, mediate resistance to lower levels (<1 g/liter) of TMP than that mediated by the other enzymes. It seems irrelevant to categorize these DHFRs by their low-level resistance phenotype in the absence of other distinguishing characteristics. The MIC conferred on the host seems to correlate rather poorly with the DHFR enzyme parameters determined in vitro (65, 77, 80), which could in part be due to specific in vivo conditions (100). There are additional genes conferring TMP resistance that have not yet been closely examined (37), and in surveys a fairly high frequency of unidentified genes is still observed (63, 64, 66, 76).

(ii) Resistance by horizontal gene transfer. The emergence of TMP resistance genes among pathogenic bacteria is very likely due to the recruitment of metabolic genes from unidentified organisms by horizontal genetic exchange. One could speculate that these genes encode the housekeeping DHFR in the cells to which they originally belong. The diversity among chromosomal DHFRs in bacteria is rather broad and includes variants with low levels of susceptibility to TMP (74, 171). TMP resistance genes use sophisticated transfer mechanisms including site-specific recombination (156, 158, 160). In fact, similarly elaborated mechanisms are involved in evolutionarily old phenomena such as phage lysogeny, variation of surface antigens, and monomerization of circular chromosomes (27).

(iii) Cassette-mediated resistance. The most widespread accessory DHFR gene among gram-negative bacteria seems to be *dhfrI*, which is commonly attributed to the successful spread of its carrier transposon, Tn*7* (20, 63, 64, 66, 76, 127, 149). This spread could be due to the capability of Tn*7* for high-frequency insertion into a preferred site in the *glmS* terminator on the *E. coli* chromosome (28, 92). Corresponding insertion sites for

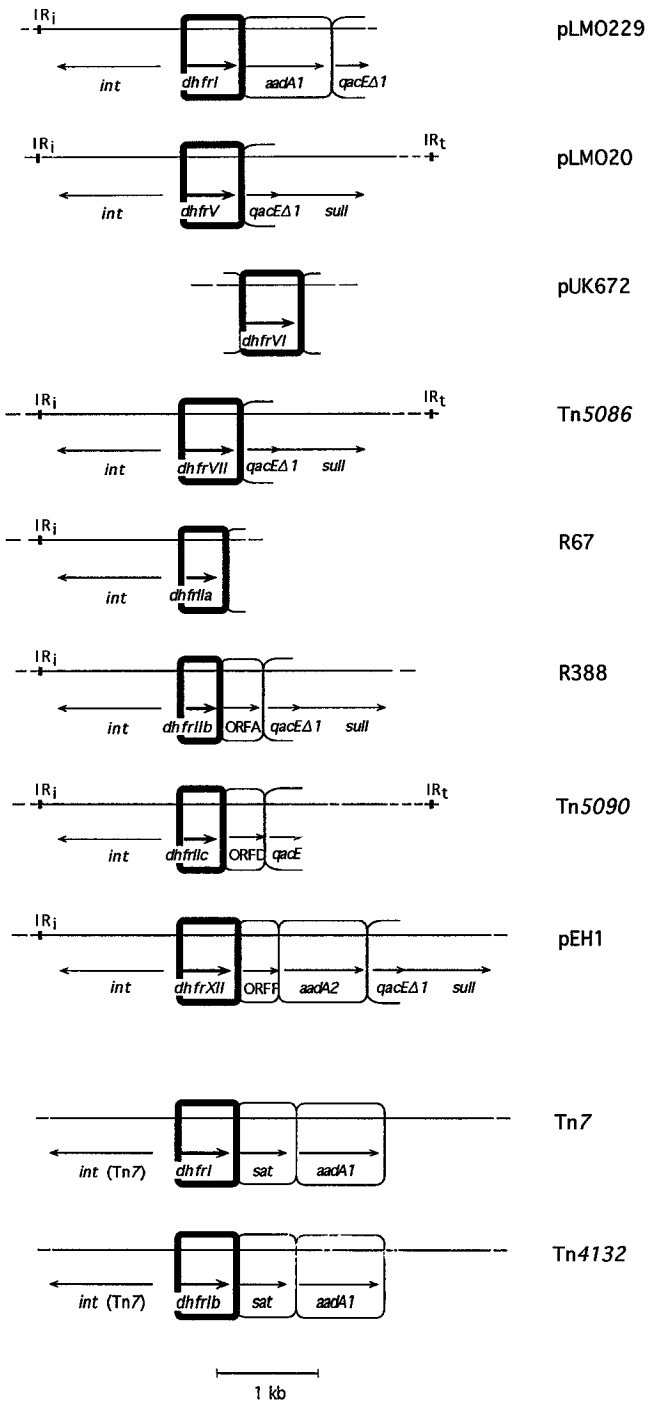

FIG. 3. Integron cassettes containing TMP resistance genes and surroundings. IR_i and IR_t refer to the 25-bp terminal repeats of Tn*5090* and related elements (129). The gene designation *int* refers to the Tn*21* type of integrase determinant. Cassettes are boxed; TMP resistance cassettes are indicated with bold lines. *qacE* codes for a multidrug resistance exporter (120).

Tn*7* have also been found in the chromosomes of a range of other bacterial species. Among clinically isolated TMP-resistant bacteria Tn*7* is, as a rule, located on the chromosome and is located less frequently on plasmids (67). The *dhfrI* gene itself is located on a type of transferable unit, called a cassette (156), which may be exchanged between different integrons (Table 1).

Because of the mobility of its cassette, *dhfrI* is also found in integrons similar to that borne on Tn*21* (159). Reciprocally, there are indications that TMP resistance genes other than *dhfrI* also occur on Tn*7*-like transposons (66, 76, 191).

The integrons are recombination systems operating to alter the contents of accessory genes among microorganisms (Fig. 3) (19, 114, 150, 156, 158). The integrons consist of one or more inserted cassettes and an adjacent gene coding for a recombinase of the integrase family (115, 156). The cassettes are flanked by GTTPuPu (where Pu is a purine) and consist of a structural gene and short inverted repeats with low or moderate levels of conservation (160). The uptake of new genes is effected by the integrase (24, 97, 155). The integrase family of proteins is rather diverse and contains two weakly conserved domains with four invariable amino acids (1, 6). The lambda phage integration system is often used as a general model for integrase-dependent site-specific recombination. In lambda integration, and similarly at its excision, the DNA strands are broken and rejoined in a sequential manner (84). A short stretch of absolute homology in the core regions of the attachment sites and the lack of a requirement for DNA repair synthesis (conservativeness) discriminate site-specific recombination from transposition. The GTTPuPu at the ends of integron cassettes is supposed to be the homologous cross-over region (25, 97, 157).

Integrons have been observed in a great variety of genetic contexts, which is due to the migration of the transposons carrying integrons, e.g., Tn*5090* and Tn*7* (129). End-truncated or otherwise incomplete variants of Tn*5090* are widespread and are commonly borne on other vehicles. One example is the Tn*21* family, which harbors a Tn*5090*-like integron-carrying element next to an operon for mercuric ion resistance (15).

Two of the first observed integron cassettes were those carrying the TMP resistance genes *dhfrIIb* and *dhfrV* (156, 158). Now, nine different types of TMP resistance cassettes have been found, which is a large fraction of the total number of resistance cassettes observed in integrons (154, 160, 191). The cassette-borne TMP resistance genes seem to be more widespread than those that are not borne on cassettes. All members of family 1 (160, 191) occur in cassettes, as do all genes of family 2 (Table 1) (129, 154). The *dhfrXII* gene, finally, has also been found in a cassette (65), whereas the *dhfrX* gene is located in the integron of plasmid pDGO100 but not on a cassette (117, 151). For the detection of integrons, probes selective for the integron integrase genes can be used (67, 159). By use of PCR it is possible to map the contents or configurations of cassettes among integrons (65, 67, 91).

Transposon Tn*7* carries the second of the two known variants of integrons. It harbors three site-specifically inserted cassettes in tandem (156, 157, 159). The first of these contains *dhfrI*, while the second and third cassettes contain genes for streptothricin acetyltransferase (*sat*) and streptomycin/spectinomycin adenylyltransferase (*aadA1*), respectively (156). The integrase gene of the Tn*7* integron is about 50% identical to that of Tn*21*, but it carries an internal stop codon (121, 139, 156). It is not known whether the integrase gene of Tn*7* is active, and its involvement in cassette exchange has not yet been proven (56, 57). There are other Tn*7*-like transposons carrying different cassettes. Tn*4132*, for instance, has recently been shown to carry another type of DHFR gene, *dhfrIb*, which forms a precise substitution of *dhfrI* in Tn*7* (191). The *dhfrIb* gene is closely related to *dhfrV* (88% identical amino acids).

(iv) Noncassette TMP resistance. Small broad-host-range plasmids are ubiquitous and frequently carry resistance to sulfonamides (*sulII*) and aminoglycosides (*strAB*) (130). Among the TMP resistance genes only *dhfrIII* has been observed in this context (41). Although the *dhfrIII* gene of plasmid pAZ1 is located in a tight cluster of genes, the mechanism by which individual genes may be added or deleted from the cluster is unknown (131).

Tn*5091* is the only known example of an IS*26*-flanked element harboring a TMP resistance gene, *dhfrVIII* (74, 154, 154b). This gene was observed by PCR mapping to be borne on Tn*5091* in several independent isolates, suggesting that it is generally spread by this element. IS*26* belongs to a family of insertion sequences which are presumed to move exclusively by cointegrate formation (44, 104). Several composite transposons carrying directly repeated IS*26* modules at the flanks have been described (96). The S1 gene in *S. aureus* is also borne in a composite transposon, Tn*4003*, flanked by IS*257* (135).

Another type of transposon mechanism for the spread of TMP resistance was recently observed among diarrheal strains of *E. coli* of piglets (77). The gene, *dhfrIX*, occurred at a significant rate among these isolates but only rarely among *E. coli* isolated from humans (75, 76). The *dhfrIX* gene is borne on Tn*3*-type transposons with a close relation to Tn*5393* from *Erwinia* sp. (22, 74). The *dhfrIX* gene and the nearby transposon end form a segment that is repeated one or several times. There is good evidence that Tn*5093*-related transposons carry *dhfrIX* in a variety of plasmids (74).

The molecular dissemination mechanisms for *dhfrIIIb* and *dhfrIIIc* (9, 174) are unknown. The induction reported for *dhfrIV* (175, 190) could be due to the action of TMP on the autoregulation of DHFR (145).

Plasmid-borne SUL resistance. Clinically occurring SUL resistance in gram-negative enteric bacteria is largely plasmid borne and is due to the presence of alternative drug-resistant variants of the DHPS enzymes (3, 142, 165, 185). Two such plasmid-encoded enzymes have been characterized, and the nucleotide sequences of the two respective genes have been determined (Table 1) (130, 156, 166). The two types of DHPS encoded by *sulI* and *sulII* show 57% amino acid identity. Both have substantial sequence divergence compared with those of chromosomal *dhps* genes from *E. coli* and other bacteria. The *sulI* gene is normally found linked to other resistance genes and is located on transposons of the Tn*21* family. A variation called *sulIII* was identified in *Mycobacterium fortuitum* (96). This gene is essentially *sulI*, but a deletion has removed the start codon and has joined the gene to another start site farther upstream (96). As a result four amino acids are added to the N terminus of the protein. The *sulII* gene is usually found on small plasmids belonging to the IncQ family (RSF1010) and also on plasmids of another type represented by pBP1 (178). Some conjugative plasmids, e.g., pGS05, carry *sulII* (130, 161, 166). The *sulI* and *sulII* genes are often found at roughly the same frequency (50% each) among SUL-resistant gram-negative clinical isolates (131).

CONCLUSIONS

TMP alone or in combination with a SUL is an effective and inexpensive antibacterial remedy. The last few years, however, have seen a dramatic increase in TMP resistance against a background of high SUL resistance. The mechanisms of resistance and of its spread among pathogenic bacteria show a remarkable evolutionary adaptation to the presence of TMP and SUL. This is reflected in the chromosomal pattern of changes in the structures and mechanisms of regulation of the *dhps* and *dhfr* genes coding for the target enzymes DHPS and DHFR, respectively.

The number of plasmid-encoded, drug-insusceptible DHFRs appearing in pathogenic bacteria is constantly increasing, and most of the genes for these enzymes characterized so far are spread by the integron mechanism. It is interesting that although the number of defined transferable genes for drug-resistant DHFRs is high and increasing, all plasmid-borne resistance to SUL in clinical isolates studied can be accounted for by *sulI* and *sulII*. Even though the use of SUL in human medicine has diminished, the genetic determinants for SUL resistance are still very common in plasmids of gram-negative bacteria, suggesting non-SUL selection factors. Alternatively, persistence can be explained by the fact that they are incorporated in very efficient vehicles for their spread; the integrons usually carry the *sulI* gene, and small multicopy plasmids carry the *sulII* gene. The common occurrence of these SUL resistance genes in genetic linkage with TMP resistance genes also largely invalidate the argument for using the TMP-SUL combination to prevent the development of resistance. In view of the molecular genetics of TMP and SUL resistance, there are no signs that removal of TMP or SUL selection pressure will have an immediate impact on the level of resistance.

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